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14. ABSTRACT

The contract supports studies to define the role of the PIM1 kinase in acquired resistance to chemotherapy by prostate cancer cells. Data to date for specific aim #1 define a signaling pathway induced by docetaxel, involving sequential steps of JAK1/2 activation, STAT3 phosphorylation, expression of PIM1, and activation of NFkB signaling. Blockade of this pathway by expression of dominant negative PIM1 proteins blocks drug-induced upregulation of NFkB activity, and sensitizes cells to docetaxel. Other studies (specific aim #2) focus on identifying a mechanism through which PIM1 activates NFkB. We have unambiguously identified S937 as the major PIM1 phosphorylation site on the NFKB1/p105 precursor protein, through use of LCM/MS/MS analysis. Other kinases that can phosphorylate this site include AKT and PKA. Additional data (specific aim #3) have been published to describe a small molecule inhibitor of PIM1. This molecule can sensitize prostate cancer cells to the cytotoxic effects of docetaxel in an additive or synergistic manner.

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INTRODUCTION

Studies under this funded activity are focused on characterizing the role of the PIM1 gene in acquired resistance to chemotherapy drugs, by prostate cancer cells. The proposal included three specific aims: 1) to define a novel signal transduction pathway activated by docetaxel, 2) to characterize the mechanism through which PIM1 activates and regulates NFkB signaling, and 3) to explore genetic and pharmacologic means of inhibiting PIM1 activity or expression to enhance the sensitivity of prostate cancer cells to docetaxel and other chemotherapy drugs. Substantial progress has been made in each of these areas during the 01 year of support.

BODY

We will outline our progress through reference to the specific aims described above. The first specific aim was to outline a signal transduction pathway activated by docetaxel and involving upregulation of PIM1 expression. This pathway has been substantially defined. Using RWPE2 prostate cells, we noted that docetaxel treatment rapidly leads to an increase in expression of the PIM1 serine/threonine kinase. Expression becomes apparent at 3hrs after drug addition, peaks at 9-12hrs, and returns to baseline by 24hrs (Fig. 1). This increase in expression is accompanied by an increase in *pim-1* mRNA, as shown by real time-PCR analysis (Fig. 2). Thus the effects of docetaxel are primarily transcriptional or post-transcriptional.

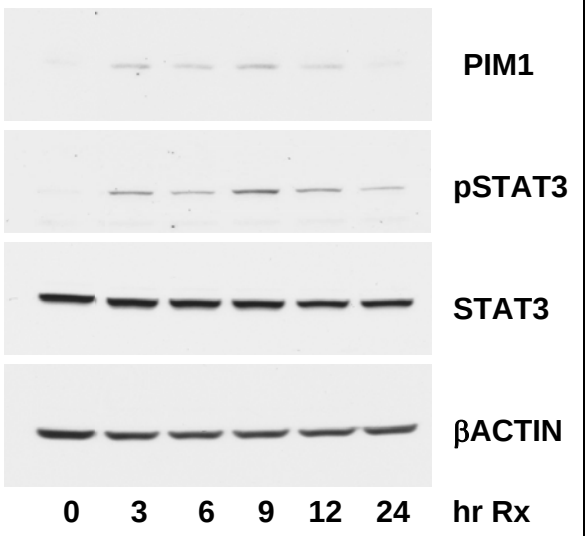
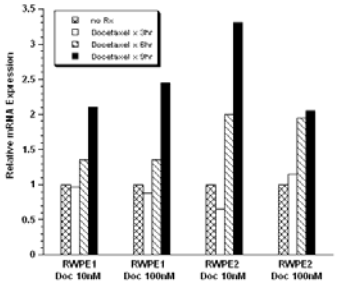


Fig. 1. Time course of PIM1, pSTAT3 expression

We next wanted to define mechanisms through which *pim-1* could be transcriptionally upregulated. Transcription of *pim-1* is known to be activated by STAT transcription factors and by NFkB transcription factors. We examined the time course of STAT3 activation after docetaxel treatment (Fig. 1), and noted that it paralleled the course of *pim-1* expression. We therefore suspected that docetaxel increased *pim-1* expression in a STAT3-dependent manner. This was directly demonstrated by use of decoy oligonucleotides (Fig. 3). Double-stranded DNA oligonucleotides matching a known STAT3 binding site blocked the drug-induced upregulation of *pim-1* expression, while a decoy based on a mutated (non-binding) STAT3 site did not. These data therefore establish a linear relationship among the following events: docetaxel treatment→ STAT3 activation→ *pim-1* expression.

Fig. 2. Expression of pim-1 mRNA after docetaxel treatment



We hypothesized that NFkB transcriptional activation would be a downstream event in this signal transduction pathway, because many chemotherapy drugs and other stressors are known to activate NFkB. We engineered RWPE2 cells to constitutively express a NFkB-dependent promoter/luciferase plasmid, and

found that docetaxel treatment increased NFkB transcriptional activity. We then transiently infected these cells with a *pim-1*-encoding retrovirus. *Pim-1* expression also consistently increased NFkB transcriptional activity (Fig. 4). To determine if the drug-induced increase in NFkB activity occurred in a *pim-1*-dependent manner, we then infected the reporter cell line with a retrovirus encoding a dominant-negative form of *pim-1*, pimNT81. The dominant negative *pim-1* cDNA completely blocked the drug-induced upregulation of NFkB activity, demonstrating that *pim-1* expression is a necessary upstream step in the drug-induced activation of NFkB (Fig. 5). In aggregate these studies establish a signal transduction pathway triggered by docetaxel treatment of RWPE2 prostate cancer cells.

Fig. 3. STAT3 decoy oligonucleotide blocks pim1 increase after docetaxel treatment

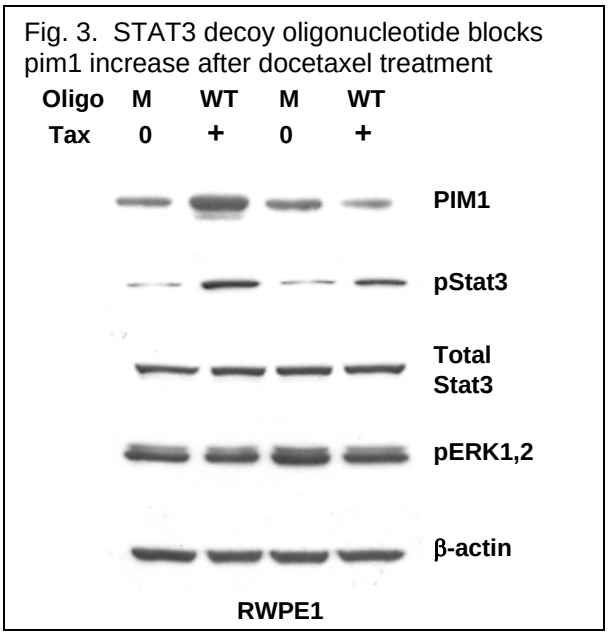


Fig. 4. Pim1 transduction increases NFkB transcriptional activity

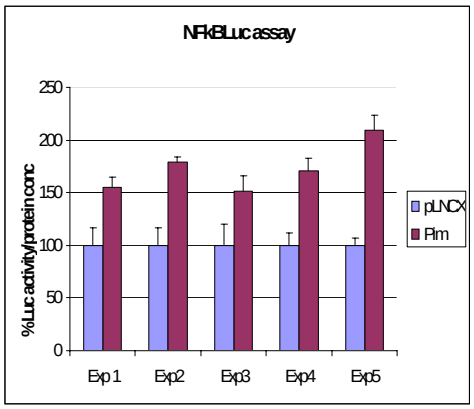
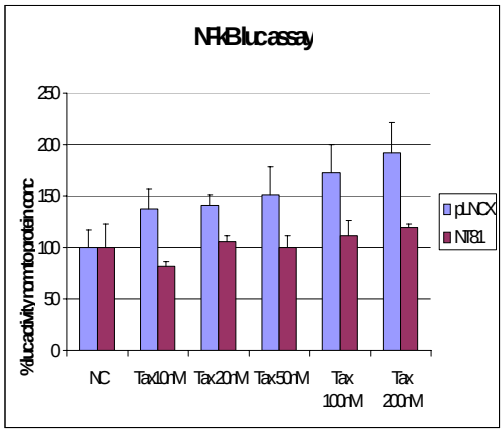
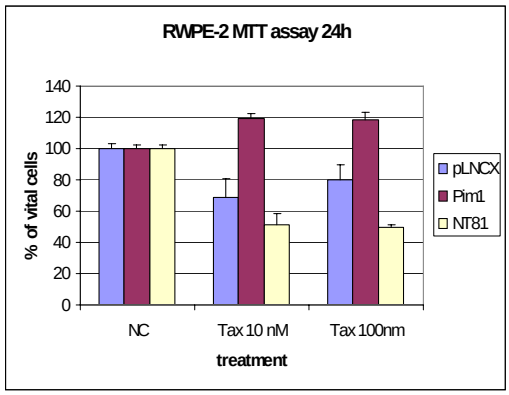


Fig. 5. Dominant negative PIM1 (NT81) blocks docetaxel-induced activation of NFkB transcriptional activity



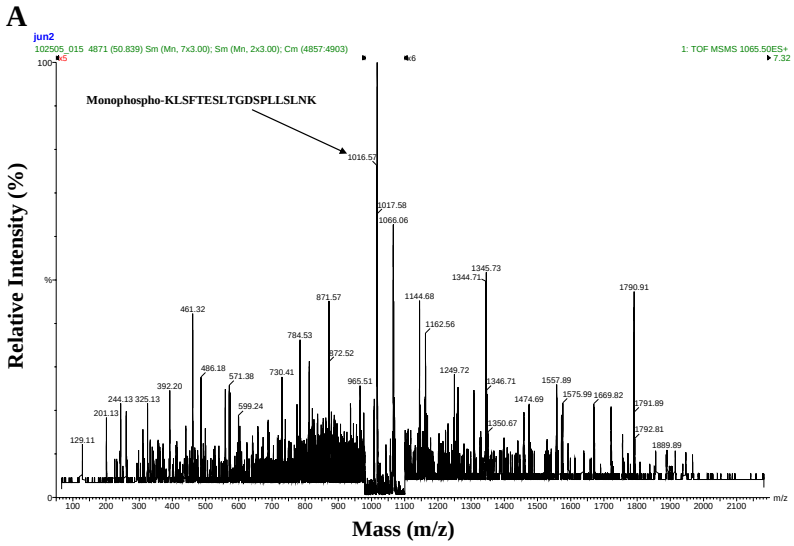
To determine if this pathway modified drug toxicity, we examined the effects of enforced expression of wild-type or NT81 *pim-1* cDNAs of docetaxel cell kill (Fig. 6). Docetaxel produced dose-dependent cell kill in RWPE1, 2 cells. Enforced expression of wild-type *pim-1* cDNA markedly reduced cell death. In contrast, expression of the dominant negative NT81 cDNA enhanced cell death after docetaxel treatment. These data demonstrate that *pim-1* expression can modulate drug-induced cell death, and demonstrate that the survival pathway described above is a legitimate target for pharmacologic intervention. These data will be presented at the 2006 AACR meeting in poster form (1).

Fig. 6. Modulation of docetaxel cell kill by enforced expression of *pim-1* cDNAs.



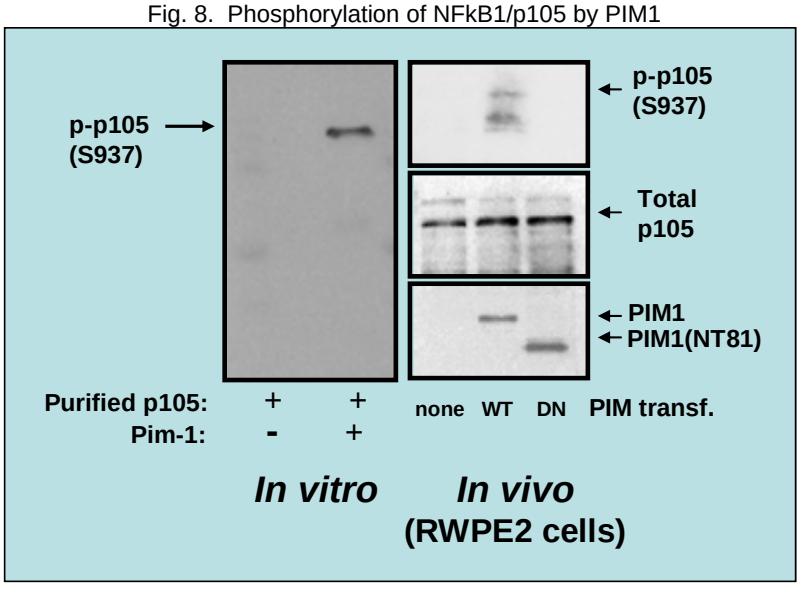
The goal of specific aim #2 was to define pathways through which the PIM1 kinase could activate NFkB transcriptional activity. We had hypothesized that PIM1 would phosphorylate the NFKB1/p105 precursor protein on serine-937, leading to proteolytic cleavage of the protein with release of active p50 protein as well as other sequestered NFkB components and the TPL2 kinase. To demonstrate the site of phosphorylation we used mass spectroscopy of tyrpsin-digested fragments of p105 that had been phosphorylated in vitro. We had previously demonstrated that PIM1-dependent phosphorylation happens exclusively on serine. Fragments were separated by LC/MS/MS analysis and mass/charge rations were determined. The predicted peptide fragment that would result from phosphorylation at serine-937 was recovered, with a mass of 1016 (Fig. 7). Since there are several potential phosphorylation sites within this peptide, we proceeded to sequence the peptide with mass spectroscopy. Only the fragment corresponding to phosphoserine-937 was not recovered. These data unambiguously demonstrate that the major phosphorylation site of PIM1 on p105 is serine-937.

Fig. 7. Mass spectroscopy of p105 phosphorylated by PIM1



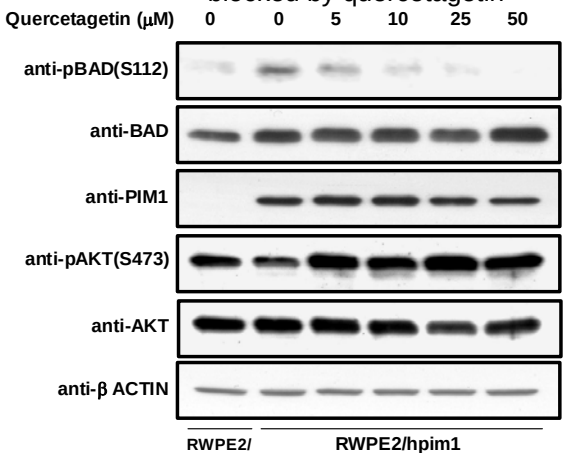
To further characterize phosphorylation on this site we prepared an antibody specific for phosphoNFkB1/p105(S937). This antibody reacts quite specifically with its antigen in whole cell lysates and immunoprecipitates. Fig. 8 demonstrates that recombinant GST-PIM1 protein phosphorylates NFkB1/p105 on serine 937 *in vitro.* Furthermore wild-type PIM1 phosphorylates p105 *in vivo*, while a dominant-negative PIM1 (NT81) does not.

Fig. 8. Phosphorylation of NFkB1/p105 by PIM1



The third specific aim proposed to use small molecule inhibitors of the PIM1 kinase as molecular probes to determine their effect on docetaxel sensitivity. A report describing one such molecule, the flavonol quercetagetin, has been accepted for publication and will appear in the January, 2007 issue of *Molecular Cancer Therapeutics* (see appendix A and Fig. 9). We have demonstrated that quercetagetin in a moderately potent (IC_{50} = 340nM, specific, and cell-permeable inhibitor of PIM1 activity in prostate cancer cells. Key data include the demonstration that quercetagetin in competitive with ATP. A crystal structure of PIM1 in complex with quercetagetin, or with three other flavonoids, has been determined. We have also shown that quercetagetin is able to inhibit the activity of the PIM1 kinase in prostate cancer cells at an IC_{50} of about 5.5μM. Interestingly the activity of the AKT kinase is not inhibited at all under these conditions (Fig. 9).

Fig. 9. PIM1 kinase activity in RWPE2/hpim1 cells is blocked by quercetagetin



We have recently obtained, and begun characterizing, novel small molecule inhibitors of PIM1 from Excelixis Corporation. These molecules show additive, or at some concentrations synergistic, cell growth inhibition in combination with docetaxel. These studies confirm the central hypothesis of this overall project, that PIM1 kinase acts to inhibit cell death caused by the cytotoxic drug docetaxel, and that blocking the activity of PIM can potentiate cell kill and overcome cytotoxic drug resistance.

KEY RESEARCH ACCOMPLISHMENTS

- Definition of a novel survival pathway activated by docetaxel treatment, and involving sequential activation or expression of JAK2, STAT3, PIM1, and NFkB components.
- Identification of serine-937 as the major phosphorylation site for PIM1 on the p105/NFKB1 precursor protein
- Identication of quercetagetin as a moderately potent and specific, cell-permeable PIM1 kinase inhibitor
- Demonstration that XL-1075 and XL-1154 can show additive or synergistic cell kill in prostate cancer cells treated with docetaxel
- Abstract presented at the annual AACR meeting, Washington DC, April, 2006
- Manuscript accepted for publication in *Molecular Cancer Therapeutics*

REPORTABLE OUTCOMES

None in 02 year

CONCLUSIONS

Our data demonstrate that PIM1 is a critical component of a survival/stress pathway activated by docetaxel treatment of prostate cancer cells. This pathway leads to activation of NFkB-dependent transcription, possibly by phosphorylation of p105/NFKB1 by PIM1 at serine-937.

Targeting PIM1 kinase activity with quercetagetin, or other PIM1 kinase inhibitors, leads to additive or synergistic cell kill following docetaxel treatment.

REFERENCES

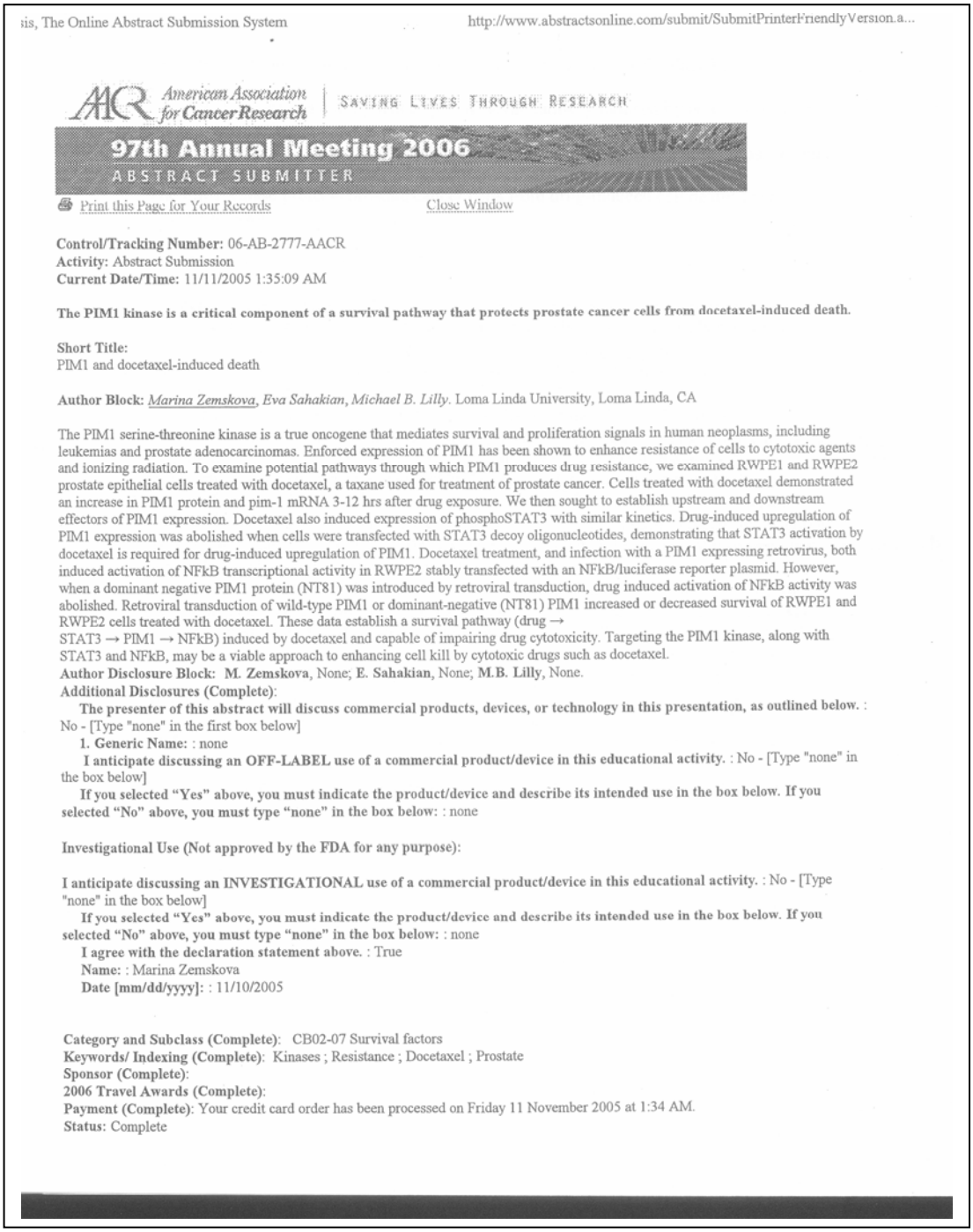
1. Zemskova M, Sahakian E, Lilly M: The PIM1 kinase is a critical component of a survival pathway that protects prostate cancer cells from docetaxel-induced death (abstract #2777), presentated at 97th Annual Meeting of AACR, Washington, DC, April 2006.

2. Holder S, Zemskova M, Bremer R, Neidigh JW, Lilly MB: Characterization of a potent and selective small-molecule inhibitor of the PIM1 kinase (in press *Molecular Cancer Therapeutics*, 2006).

APPENDIX

Research data are presented throughout the body of this report.
The appendix contains three items:

1. AACR abstract #2777, approved for presentation at the 97th Annual Meeting, April, 2006, entitled "The PIM1 kinase is a critical component of a survival pathway that protects prostate cancer cells from docetaxel-induced death" by M. Zemskova, E. Sahakian, M. Lilly.
2. Galley proofs of manuscript accepted for *Molecular Cancer Therapeutics*
2. Curriculum vitae for Michael Lilly, MD



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The PIM1 kinase is a critical component of a survival pathway that protects prostate cancer cells from docetaxel-induced death.

Short Title:
PIM1 and docetaxel-induced death

Author Block: *Marina Zemskova, Eva Sahakian, Michael B. Lilly.* Loma Linda University, Loma Linda, CA

The PIM1 serine-threonine kinase is a true oncogene that mediates survival and proliferation signals in human neoplasms, including leukemias and prostate adenocarcinomas. Enforced expression of PIM1 has been shown to enhance resistance of cells to cytotoxic agents and ionizing radiation. To examine potential pathways through which PIM1 produces drug resistance, we examined RWPE1 and RWPE2 prostate epithelial cells treated with docetaxel, a taxane used for treatment of prostate cancer. Cells treated with docetaxel demonstrated an increase in PIM1 protein and pim-1 mRNA 3-12 hrs after drug exposure. We then sought to establish upstream and downstream effectors of PIM1 expression. Docetaxel also induced expression of phosphoSTAT3 with similar kinetics. Drug-induced upregulation of PIM1 expression was abolished when cells were transfected with STAT3 decoy oligonucleotides, demonstrating that STAT3 activation by docetaxel is required for drug-induced upregulation of PIM1. Docetaxel treatment, and infection with a PIM1 expressing retrovirus, both induced activation of NFkB transcriptional activity in RWPE2 stably transfected with an NFkB/luciferase reporter plasmid. However, when a dominant negative PIM1 protein (NT81) was introduced by retroviral transduction, drug induced activation of NFkB activity was abolished. Retroviral transduction of wild-type PIM1 or dominant-negative (NT81) PIM1 increased or decreased survival of RWPE1 and RWPE2 cells treated with docetaxel. These data establish a survival pathway (drug → STAT3 → PIM1 → NFkB) induced by docetaxel and capable of impairing drug cytotoxicity. Targeting the PIM1 kinase, along with STAT3 and NFkB, may be a viable approach to enhancing cell kill by cytotoxic drugs such as docetaxel.
Author Disclosure Block: M. Zemskova, None; **E. Sahakian**, None; **M.B. Lilly**, None.
Additional Disclosures (Complete):
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I agree with the declaration statement above. : True
Name: : Marina Zemskova
Date [mm/dd/yyyy]: : 11/10/2005

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Keywords/ Indexing (Complete): Kinases ; Resistance ; Docetaxel ; Prostate
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Characterization of a potent and selective small-molecule inhibitor of the PIM1 kinase

Sheldon Holder,[1,2,3] Marina Zemskova,[2] Chao Zhang,[4] Maryam Tabrizizad,[4] Ryan Bremer,[4] Jonathan W. Neidigh,[2] and Michael B. Lilly[1,2,3]

[1]Center for Molecular Biology and Gene Therapy, Departments of [2]Biochemistry and Microbiology and [3]Medicine, Loma Linda University School of Medicine, Loma Linda, California; and [4]Hexadion, Inc., Berkeley, California

Abstract

Q2 The *pim-1* kinase is a true oncogene that has been implicated in the development of leukemias, lymphomas, and prostate cancer, and is the target of drug development programs. We have used experimental approaches to identify a selective, cell-permeable, small-molecule inhibitor of the *pim-1* kinase to foster basic and translational studies of the enzyme. We used an ELISA-based kinase assay to screen a diversity library of potential kinase inhibitors. The flavonol *quercetagetin* (3,3′,4′,5,6,7-hydroxyflavone) was identified as a moderately potent, ATP-competitive inhibitor (IC_{50}, 0.34 μmol/L). Resolution of the crystal structure of PIM1 in complex with quercetagetin or two other flavonoids revealed a spectrum of binding poses and hydrogen-bonding patterns in spite of strong similarity of the ligands. Quercetagetin was a highly selective inhibitor of PIM1 compared with PIM2 and seven other serine-threonine kinases. Quercetagetin was able to inhibit PIM1 activity in intact RWPE2 prostate cancer cells in a dose-dependent manner (ED_{50}, 5.5 μmol/L). RWPE2 cells treated with quercetagetin showed pronounced growth inhibition at inhibitor concentrations that blocked *pim-1* kinase activity. Furthermore, the ability of quercetagetin to inhibit the growth of other prostate epithelial cell lines varied in proportion to their levels of PIM1 protein. Quercetagetin can function as a moderately potent and selective, cell-permeable inhibitor of the *pim-1* kinase, and may be useful for proof-of-concept studies to support the development of clinically useful PIM1 inhibitors. [Mol Cancer Ther 2007;6(1):1–10]

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Introduction

The *pim* family of serine-threonine kinases is composed of three highly homologous genes, *pim-1*, *pim-2*, and *pim-3*. These enzymes are increasingly being recognized as important mediators of survival signals in cancers, stress responses, and neural development (1–6). In addition, these kinases are constitutively expressed in some tumors and function as true oncogenes. Thus, they are of significant interest as targets for therapeutic intervention.

Small-molecule inhibitors are important molecular probes for studying protein kinases. In addition, they may serve as protype therapeutic agents for treating diseases resulting from unregulated kinase activity. Three prior reports have shown that known, promiscuous kinase inhibitors can inhibit PIM1 function *in vitro*. Jacobs et al. (7) showed that several staurosporine and bisindolyl-maleimide analogues, as well as the morpholino-substituted chromone LY294002, were able to inhibit PIM1 activity *in vitro*. Subsequently, Fabian et al. (8) presented an interaction map involving 113 kinases and 20 small-molecule kinase inhibitors now under clinical study. Only three inhibitors had detectable binding to (and presumably inhibitory activity against) PIM1—two staurosporine analogues and flavopiridol, a flavonoid undergoing evaluation as an inhibitor of cyclin-dependent kinases. A recent report (9) confirmed the activity of bisindolylmaleimide derivatives as well as some flavonoids *in vitro*. All of the identified inhibitors either lacked specificity for PIM1 or were only modestly active at low micromolar concentrations, or both. Furthermore, none of these reports showed that the test agents could selectively inhibit PIM1 activity in intact cells.

To further our basic and translational studies of the *pim* kinases, we have sought to identify small-molecule inhibitors of PIM1. We here report that the flavonol *quercetagetin* is a selective PIM1 inhibitor with nanomolar potency and can differentially inhibit the kinase in cell-based assays.

Materials and Methods

Cell Lines and Culture Methods

The prostate epithelial cell lines RWPE1, RWPE2, LNCaP, and PC3 were obtained from the American Type Culture Collection (Manassas, VA) and cultured in the recommended medium. We produced additional pools of RWPE2 prostate cells that overexpressed *pim-1* through retroviral transduction. The coding region for the human *pim-1* gene was cloned into the pLNCX retroviral vector (Clontech). Infectious viruses were produced in the GP-293 packaging cell line by cotransfection with retroviral backbone plasmids (pLNCX or pLNCX/*pim-1*) and with pVSV-G, a plasmid that expresses the envelope glycoprotein from Q3 Q4

vesicular stomatitis virus. Forty-eight hours after transduction, the medium was collected and the virus particles were concentrated as described in the manufacturer's protocol (Clontech). RWPE2 cells were plated at 1×10^5 per 60-mm plate 16 to 18 h before infection. Cells were infected with 5×10^4 viral particles in the presence of 8 μg/mL polybrene. After 6 h of incubation, the virus-containing medium was replaced with fresh medium, and on the next day G418 (400 μg/mL) was added to select infected cells. After 10 days of selection, stable cell pools were established and PIM1 expression was verified by immunoblotting.

For growth-inhibition experiments, cells were plated onto 24-well plates and fixed with formaldehyde at intervals. Cell number was quantified by crystal violet staining (10).

Recombinant *pim* Kinases and Kinase Assays

We prepared recombinant PIM1 and PIM2 as glutathione *S*-transferase (GST) fusions in *Escherichia coli*, as described (11). For the inhibitor screening assays, a solid-phase kinase assay was developed based on our demonstration that PIM1 is a potent kinase for phosphorylating BAD on Ser^{112} (11, 12). Ninety-six-well flat-bottomed plates (Corning) were coated overnight at 4°C with recombinant GST-BAD [1 μg/well in HEPES buffer: 136 mmol/L NaCl, 2.6 mmol/L KCl, and 20 mmol/L HEPES (pH 7.5)]. The plates were then blocked for 1 h at room temperature with 10 mg/mL bovine serum albumin in HEPES buffer. The blocking solution was then removed and 5 μL of each inhibitor, dissolved in 50% DMSO, were added to each well. Then, 100 μL of kinase buffer [20 mmol/L MOPS (pH 7.0), 12.5 mmol/L $MgCl_2$, 1 mmol/L $MnCl_2$, 1 mmol/L EGTA, 150 mmol/L NaCl, 10 μmol/L ATP, 1 mmol/L DTT, and 5 mmol/L β-glycerophosphate] containing 25 ng recombinant GST-PIM1 kinase were added to each well. The final concentration of each inhibitor was ~10 μmol/L. The plate was placed on a gel slab dryer prewarmed to 30°C, and the kinase reaction was allowed to proceed. The reaction was stopped after 60 min by removal of the reaction buffer, followed by the addition of 100 μL of HEPES buffer containing 20 mmol/L EDTA to each well. Phosphorylated GST-BAD was detected by an ELISA reaction, using as first antibody a monoclonal anti–phospho-BAD(S112) antibody (Cell Signaling), a secondary goat anti-mouse IgG-peroxidase conjugated antibody (Pierce), and Turbo-TMB peroxidase substrate (Pierce). The level of phosphorylated GST-BAD present was proportional to the absorbance at 450 nm.

For quantitative and kinetic studies of inhibitors against various BAD(S112) kinases, a solution phase assay was used. A biotinylated peptide based on the PIM1 phosphorylation site of human BAD was synthesized (GGAGAVEIRSRHSSYPAGTE) and used as the assay substrate. Recombinant GST-PIM1 (25 ng/reaction) was preincubated with various concentrations of inhibitors in the previous kinase buffer (final volume 100 μL). The reaction proceeded by addition of substrate peptide, followed by incubation for 5 min in a 30°C water bath. The reaction was terminated by transferring the mixture to a streptavidin-coated 96-well plate (Pierce) containing 100 μL/well of 40 mmol/L EDTA. The biotinylated peptide substrate was allowed to bind to the plate at room temperature for 10 min. The level of phosphorylation was then determined by ELISA as described above. Curve fitting and enzyme analyses were done using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA). For the additional BAD(S112) kinases [PIM2, RSK2 (ribosomal S6 kinase 2), and PKA (cyclic AMP–dependent protein kinase)], reaction components were as described above. As with the PIM1 assays, an ATP concentration of 10 μmol/L was used. Furthermore, with each kinase, linear reaction velocities for the duration of the reaction were confirmed (data not shown).

To further assess the specificity of quercetagetin as a PIM1 inhibitor, its activity against a panel of serine-threonine kinases was also studied through a commercial kinase inhibitor profiling service (KinaseProfiler; Upstate Biotechnology). All KinaseProfiler assays were conducted using 10 μmol/L ATP concentrations.

Small-Molecule Library Screening

We obtained a library of 1,200 compounds that had structural affinity to known kinase inhibitors (TimTec, Inc.). The entire library was screened once with our solid-phase ELISA kinase assay, with each compound at ~10 μmol/L concentration. Positive hits were rescreened at the same concentration. Compounds that had reproducible activity at 10 μmol/L were then screened at a range of concentrations from 0.001 to 300 μmol/L. Additional flavonoids were purchased from Indofine Chemicals and were tested in a similar protocol.

Measurement of PIM1 Kinase Activity in Cells

RWPE2 cell pools, stably infected with empty retrovirus or *pim-1*–encoding retrovirus, were seeded in six-well plates at 5×10^5 cells per well. After 18 h, the normal supplemented keratinocyte medium was removed and replaced with supplement-free keratinocyte medium. Cells were then incubated for an additional 20 h. Quercetagetin, or an equivalent volume of DMSO, was added to the cells 3 h before the end of the starvation period. At the conclusion of the starvation period, the cells were washed twice with PBS and subsequently lysed in a denaturing buffer with protease, phosphatase inhibitors. The lysates were normalized by total protein content (BCA protein assay, Pierce), then analyzed by immunoblotting with the following antibodies: monoclonal anti-PIM1 (Santa Cruz Biotechnologies, Santa Cruz, CA); monoclonal anti–β-actin (Sigma); monoclonal anti-BAD (Transduction Laboratories); and monoclonal anti–phospho-BAD(S112), polyclonal anti–phospho-AKT(S473), and anti-AKT (all from Cell Signaling).

Cloning, Expression, Purification, and Crystallization of PIM1

The production, purification, and characterization of recombinant 6His-tagged PIM1 proteins for crystallography have been described previously (13). To obtain cocrystals of complexes of the protein with ligands, the protein solution was initially mixed with the compound (dissolved in DMSO) at a final compound concentration of 1 mmol/L and then set up for crystallization. The protein was

crystallized by a sitting-drop, vapor-diffusion experiment in which equal volumes of protein (10–15 mg/mL concentration) and reservoir solution [0.4–0.9 mol/L sodium acetate, 0.1 mol/L imidazole (pH 6.5)] were mixed and allowed to equilibrate against the reservoir at 4°C. The crystals routinely grew to a size of 200 × 200 × 800 μm in ~2 to 3 days.

Structure Determination

X-ray diffraction data were collected at Advanced Light Source (Berkeley, CA). All data were processed and reduced with MOSFLM and scaled with SCALA of the CCP4 suite of programs using the software ELVES. The space group of all crystals was determined to be $P6_1$, with the cell axes being approximately 99, 99, and 80, and one protein monomer being present in the asymmetrical unit. All structures were determined by molecular replacement using the apo PIM1 structure (1YWV; ref. 13) as a model, and refined by CNX and REFMACS. Crystallographic statistics are reported in Supplementary Table S1.[5] The coordinates and structure factors for the structures have been deposited with the RCSB Protein Data Bank (accession codes).

Results

Screening of a Chemical Library with Structural Affinity to Known Kinase Inhibitors

As an initial approach to the identification of PIM1 inhibitors, we screened a library of small molecules whose structures were similar to those of known kinase inhibitors. Of the seven compounds that had reproducible inhibitory activity at 10 μmol/L, six were flavonoids [quercetin, luteolin, kaempferol, 7-hydroxyflavone, (*S*)-5,7-dihydroxy-8-(3-methylbut-2-enyl)flavanone, and (*R*)-5,7-dihydroxyflavanone]. These compounds exhibited a range of inhibitory potencies (as IC_{50}) from 1.1 to 60 μmol/L. Thirty-seven other flavonoids failed to show detectable inhibitory activity at 10 μmol/L. These inactive compounds were characterized in most cases by bulky (charged or uncharged) groups at the 3, 3′, 4′, or 7 positions; lack of at least two hydrogen bond donors on the A or C rings; presence of glycoside linkages; or failure of all rings to adopt a planar conformation.

The most active compound in the chemical library was the flavonol quercetin (IC_{50}, 1.1 μmol/L), a known inhibitor of kinases and many other enzymes (14–19). Furthermore, six of the seven compounds with reproducible activity at 10 μmol/L were flavonoids. Hence, we screened additional flavonoids to identify molecules with inhibitory activity against the PIM1 kinase (Fig. 1). The most active molecule was the flavonol *quercetagetin* (IC_{50}, 0.34 μmol/L). The four flavonoids with the highest inhibitory activity were characterized by the presence of five to six –OH groups distributed between the A and B rings. In comparison, the hydroxyl groups on the B ring seemed to be more critical for the activity of the compounds than those on the A ring, as compounds with an unsubstituted B ring showed greatly reduced activity. Finally, a hydrophobic substituent at the 8 position was tolerated.

Quercetagetin Is a Selective, Potent Inhibitor of PIM1 *In vitro*

To assess the selectivity of quercetagetin for PIM1, we determined its IC_{50} value toward the alternative BAD(S112) kinases RSK2, PKA, and PIM2 (Table 1). The IC_{50} of quercetagetin for PIM1 kinase was 0.34 μmol/L, whereas the corresponding values for the other kinases were 9- to 70-fold higher.

To further characterize the specificity of quercetagetin, its inhibitory activity was examined at 1 or 10 μmol/L against additional serine-threonine kinases (c-Jun-NH_2-kinase 1, PKA, Aurora-A, c-RAF, and PKC8; Fig. 2). At the lower concentration, the selectivity of quercetagetin was most apparent. In the presence of 1 μmol/L inhibitor, PIM1 activity was inhibited by 92%. In contrast, the activity of the other kinases was inhibited by only 0% to 41%. In aggregate, these studies established that quercetagetin was a sevenfold more potent inhibitor for *pim-1* kinase than for seven other serine-threonine kinases. In addition, quercetagetin was completely inactive against the *c-abl* tyrosine kinase when tested at the 200 μmol/L concentration (data not shown).

Crystallographic Analysis of Quercetagetin in Complex with PIM1

Recently, several crystal structures of the *pim-1* kinase have been solved and presented, including apo forms and the enzyme in complex with a variety of ligands (7, 9, 13, 20, 21). Because the PIM1 protein has several unique structural features around its ATP-binding pocket, including the lack of the canonical hydrogen bond donor from the hinge region typically used by kinases to bind ATP-like ligands, we determined the crystal structure for the kinase in complex with three flavonoid inhibitors: quercetagetin, myricetin, and 5,7,3′,4′,5′-pentahydroxyflavone (Fig. 3).

The three flavonoid inhibitors show two distinct binding poses, denoted here as orientations I and II, respectively. Quercetagetin, the compound with two hydroxyl groups on the B ring, adopts orientation I, whereas the compounds with a trisubstituted B ring (myricetin and 5,7,3′,4′,5′-pentahydroxyflavone) adopt orientation II.

The binding pose of quercetagetin in PIM1 (Fig. 3A) closely resembles that of quercetin in phosphatidylinositol 3-kinase γ (1E8W; ref. 22) and that of fisetin in CDK6 (1XO2; ref. 23), designated here as orientation I. As seen in the two earlier structures (Fig. 3D and E), the 3-OH of the quercetagetin (Fig. 3A) makes a canonical hydrogen bond with backbone carbonyl oxygen of the hinge residue Glu^{121}. In addition, the B ring of quercetagetin binds deep inside the PIM1 ATP-binding pocket, with the 4′-hydroxyl group hydrogen-bonded to the side chains of two highly conserved residues, Lys^{67} and Glu^{89}. However, significant difference was also observed between the current structure and the two reported structures. In both 1E8W and 1XO2, the 4-keto group of the chromenone core of the compound

[5] Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

formed a hydrogen bond with the same hinge amide nitrogen [Val[99] in phosphatidylinositol 3-kinase γ (Fig. 3D) and Val[104] in CDK6 (Fig. 3E)]. However, there is no direct interaction between the 4-keto group of quercetagetin and the amide nitrogen of the corresponding residue Pro[123] in PIM1 because proline is incapable of acting as a hydrogen bond donor. Instead, the 4-keto group of quercetagetin makes close contact with the backbone Cα of Arg[122] (3.4 Å). It is not clear whether this interaction makes a positive contribution to the binding of quercetagetin to PIM1.

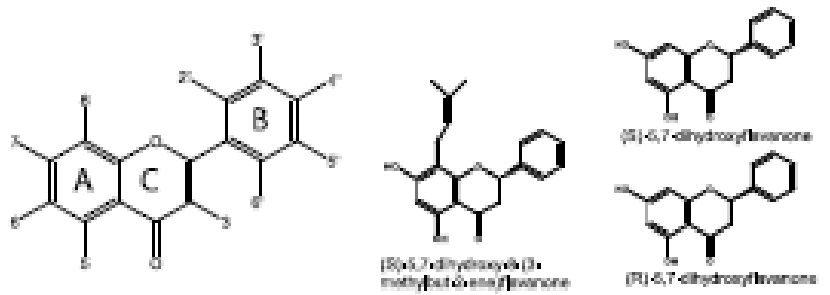
The B ring of quercetagetin binds deep inside the PIM1 ATP-binding pocket. The 4′-hydroxyl group forms hydrogen bonds with both Lys[67] and Glu[89], two of the most conserved residues in kinases. As has been noted, satisfying the hydrogen bonding requirements at this region is one of the determining features of binding of compounds to PIM1 (13).

When compared with quercetagetin, the chromenone core of myricetin (Fig. 3B) and 5,7,3′,4′,5′-pentahydroxyflavone (Fig. 3C) has flipped 180° in PIM1 such that the B ring is now oriented toward the entrance of the ATP pocket. A possible explanation for adopting this orientation is that the interior of the ATP pocket cannot accommodate the B ring with three hydroxyl substitutions. Although they bind in the same orientation, there are important differences between the binding poses of the two compounds, which can be attributed to the presence or absence of the 3-hydroxyl group. The 3-hydroxyl group in myricetin still makes a hydrogen bond with the carbonyl oxygen of Glu[121], despite the difference in binding orientation. Because of the adjacent 4-keto group, the 3-hydroxyl is likely to be most acidic of all the hydroxyl groups in the compound, and, as a result, it dictates the overall positioning of the compound. Another interaction that may contribute to the observed binding pose is a hydrogen bond between the 3′-hydroxyl group of myricetin and the carbonyl oxygen of Pro[123] (Fig. 3B). The importance of the 3-hydroxyl group is evident. The second compound, 5,7,3′,4′,5′-pentahydroxyflavone, lacking such a group, makes no direct interaction with the hinge region.

Quercetagetin Inhibits PIM1 Kinase Activity in Intact Cells

To determine if quercetagetin could act as a cell-permeable PIM1 inhibitor, we examined the activity of the flavonol in RWPE2 prostate cancer cells. We studied the phosphorylation of endogenous BAD on Ser[112], under conditions of growth factor starvation, as an indicator of intracellular PIM1 activity (Fig. 4).

RWPE2 cells infected with a neo-expressing retrovirus showed little phospho-BAD(Ser112) when cultured overnight



Flavonoid	3	5	6	7	8	2′	3′	4′	5′	6′	IC_{50} (μM)
quercetagetin	OH	OH	OH	OH	H	H	OH	OH	H	H	0.24
gossypetin	OH	OH	H	OH	OH	H	OH	OH	H	H	0.43
5,7,3′,4′,5′-pentahydroxyflavone	H	OH	H	OH	H	H	OH	OH	OH	H	0.85
myricetin	OH	OH	H	OH	H	H	OH	OH	OH	H	0.78
fisetin	OH	H	H	OH	H	H	OH	OH	H	H	0.85
apigenin	H	OH	H	OH	H	H	H	OH	H	H	0.84
3,7,4′-trihydroxyflavone	OH	H	H	OH	H	H	H	OH	H	H	0.88
quercetin	OH	OH	H	OH	H	H	OH	OH	H	H	1.1
kaempferol	OH	OH	H	OH	H	H	H	OH	H	H	1.3
luteolin	H	OH	H	OH	H	H	OH	OH	H	H	1.6
morin	OH	OH	H	OH	H	OH	H	OH	H	H	3.7
7,3′-dihydroxyflavone	H	H	H	OH	H	H	OH	H	H	H	4.52
3,7-dihydroxyflavone	OH	H	H	OH	H	H	H	H	H	H	4.6
7,3′,4′,5′-tetrahydroxyflavone	H	H	H	OH	H	H	OH	OH	OH	H	7.8
3,6,3′,4′-tetrahydroxyflavone	OH	H	OH	H	H	OH	H	OH	H	H	8.3
(S)-5,7-dihydroxy-8-(3-methylbut-2-enyl)flavanone	see illustration above										12.5
7-hydroxyflavone	H	H	H	OH	H	H	H	H	H	H	14
5,7-dihydroxyflavone	H	OH	H	OH	H	H	H	H	H	H	15
7,8,4′-trihydroxyflavone	H	H	H	OH	OH	H	H	OH	H	H	22
(R)-5,7-dihydroxyflavanone	see illustration above										60.2
(S)-5,7-dihydroxyflavanone	see illustration above										107

Figure 1. Identification of flavonoids with PIM1 inhibitory activity. Structures of all studied flavonoids with detectable PIM1 inhibitory activity, given with their IC_{50} values.

Table 1. Quercetagetin is a selective inhibitor of the PIM1 kinase over other BAD(S112) kinases

Kinase	IC_{50} (μmol/L)	Log IC_{50} (μmol/L)	SE of log IC_{50}	R^2
PIM1	0.34	−0.46	0.12	0.98
PIM2	3.48	0.55	0.22	0.94
PKA	21.2	1.33	0.23	0.94
RSK2	2.62	0.45	0.09	0.99

NOTE: All data were derived from nonlinear regression analyses using a three-parameter logistic that assumes a Hill coefficient of −1.

in basal serum-free medium. However, cells with enforced expression of *pim-1* kinase had a 4-fold higher amount of phospho-BAD, reflecting the ability of the PIM1 protein to phosphorylate the endogenous BAD protein. When *pim-1*–expressing cells were treated with quercetagetin, phospho-BAD(S112) levels were markedly reduced in proportion to the concentration of the inhibitor. Half-maximal inhibition occurred at 5.5 μmol/L extracellular concentration. Quercetagetin did not inhibit the activity of the AKT kinase under these conditions, as indicated by persistent phosphorylation of AKT on Ser^{473}. These data indicate that quercetagetin was able to selectively block the ability of PIM1 to phosphorylate BAD in intact cells.

Quercetagetin Treatment Reproduces a Known *pim-1* Knockdown Phenotype

If quercetagetin acts as a true PIM1 inhibitor, then it should reproduce a *pim-1*–dependent phenotype in the target cells. We have shown that PIM1 inhibition by genetic means (small interfering RNA) inhibits the proliferation of RWPE1 and RWPE2 cells (Supplementary Fig. S1).[8] We therefore determined if quercetagetin could reproduce this phenotype. RWPE2 cells were treated with quercetagetin for up to 72 h (Fig. 5A). Marked dose-dependent growth inhibition was apparent by 24 h, leading to persistent growth arrest thereafter. Quercetagetin reproduced this *pim-1*–dependent phenotype at a drug concentration that inhibited the enzyme in cells (ED_{50}, 3.8 μmol/L; Fig. 5B). Similar results were seen in RWPE1 cells (data not shown). Apoptotic cells, showing cytoplasmic blebbing and detachment, were rare, but dividing cells virtually disappeared in cultures treated with quercetagetin at 6.25 μmol/L or higher concentrations (data not shown). DNA histograms obtained at 24 h after the addition of quercetagetin (6.25 μmol/L) or DMSO vehicle were very similar (Fig. 5C). Neither showed a <2n population suggestive of apoptosis. There was a slight increase in the proportion of cycling cells (S + G_2-M) in the drug-treated samples.

A PIM1 inhibitor would be predicted to inhibit the growth of cells that express the molecular target, more than cells with little or no *pim-1* expression. We examined the effects of quercetagetin on the growth of prostate cell lines that express a spectrum of PIM1 levels. RWPE2 cells expressed the highest amount of PIM1 protein; PC3 had an intermediate level; and LNCaP cells showed the lowest amount of kinase protein (Fig. 6A). Treatment of the cells with various concentrations of quercetagetin for 72 h resulted in inhibition of cell growth (Fig. 6B). At all concentrations, RWPE2 cells were inhibited the most, being significantly more sensitive to quercetagetin growth inhibition than the other prostate cancer cell lines. PC3 cells showed intermediate growth suppression and were also significantly more sensitive than were LNCaP cells at quercetagetin concentrations of ≤12.5 μmol/L. Thus, the ability of the flavonol to inhibit proliferation was proportional to the amount of PIM1 protein in the target cells, particularly at lower drug concentrations. Although other interpretations are possible, these data support our observation that quercetagetin can act as a PIM1 inhibitor.

Discussion

The development of clinically useful small-molecule kinase inhibitors has been a seminal event in the world of oncology. Flavonoids were among the early scaffold structures identified as potential kinase inhibitors. However, although many flavones, isoflavones, and flavonols have been shown to regulate the activity of kinases in cell-based assays, fewer data exist to show that these molecules can directly bind and inhibit kinase targets both *in vitro* and in cells. It is clear that some flavonoids are ATP-competitive ligands for both tyrosine and serine-threonine kinases, as well as other ATP-binding enzymes. The flavonol quercetin is one such ligand, and its ability to directly bind to ATP-binding enzymes has been well shown. At low-micromolar concentrations, it directly binds and inhibits such diverse enzymes as the phosphatidylinositol 3-kinase (14), the epidermal growth factor receptor tyrosine kinase (15), retroviral reverse transcriptases (16), DNA gyrases (17), phosphodiesterases (18), and thioredoxin reductase (19). Other direct flavonoid inhibitors have been described for RSK2 kinase (24), mitogen-activated protein/extracellular signal-regulated kinase 1 (25), and several cyclin-dependent kinases (23, 26–28). One such ligand, flavopiridol, has already entered clinical trials for the treatment of cancer. Others, such as PD98059, are familiar laboratory reagents for inhibition of kinase pathways. We now show, by means of crystallography, that quercetagetin is a direct ligand for the ATP-binding pocket of PIM1 kinase (Fig. 3).

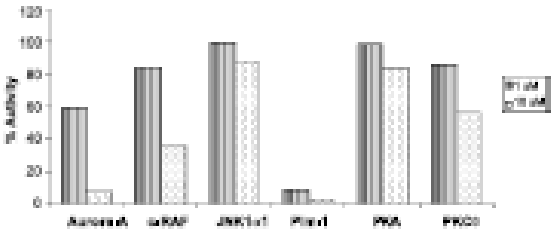


Figure 2. Quercetagetin is a selective inhibitor of PIM1 kinase. Inhibitor activity of quercetagetin at 1 and 10 μmol/L final concentration against a spectrum of serine-threonine kinases of a panel of kinases, assessed by KinaseProfiler assay. The activity in the presence of vehicle only was taken to be 100% activity. Columns, mean of duplicate determinations.

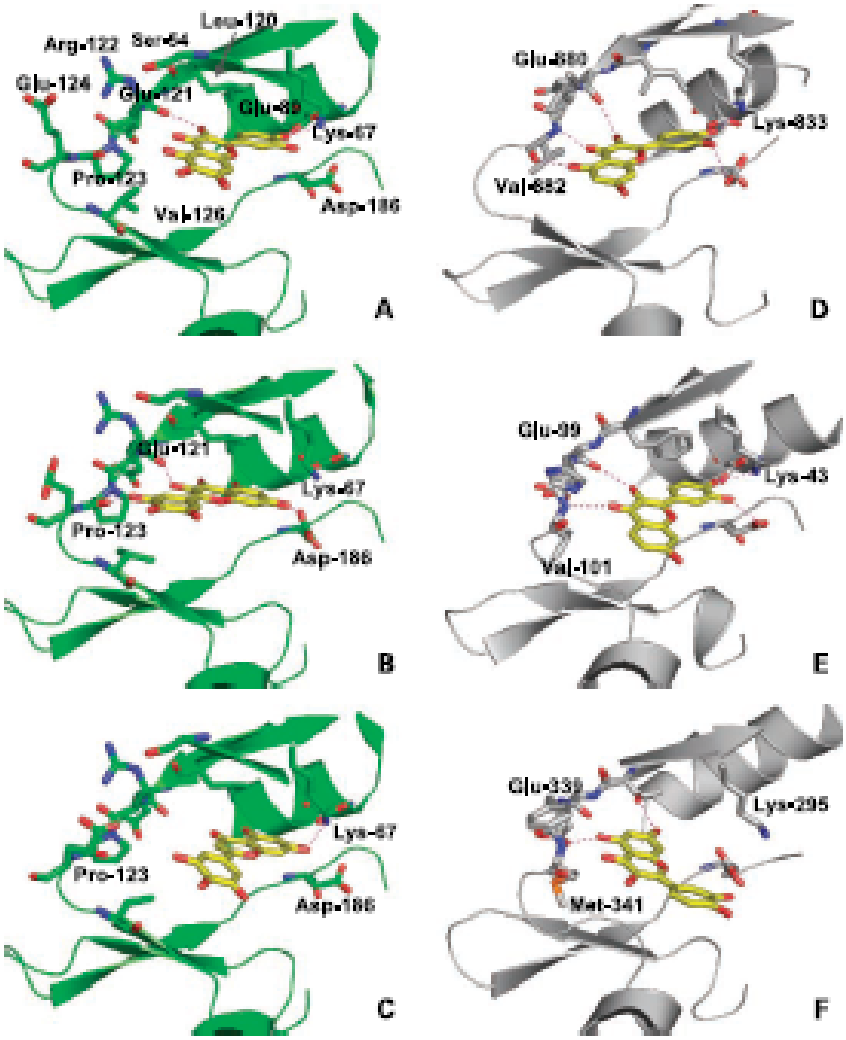


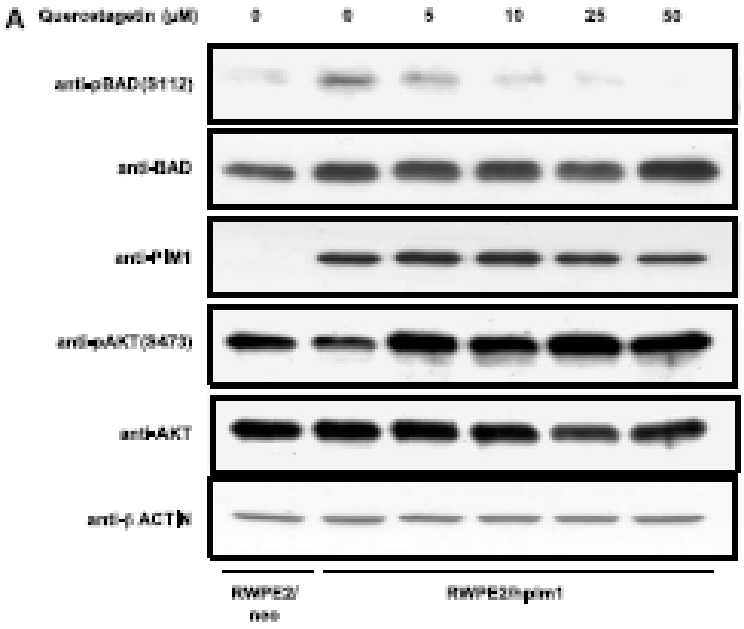
Figure 3. X-ray crystal structures of flavonoids bound to the ATP-binding sites of PIM1 and comparison with structures of flavonoids in complex with other kinases. Of the three compounds cocrystallized with PIM1, quercetagetin (*A*) orients the B ring inside the pocket (orientation I), whereas both myricetin (*B*) and 5,7,3′,4′,5′-pentahydroxyflavone (*C*) flip the B ring out toward solvent (orientation II). Both binding orientations have been observed in the crystal structures of flavonoids bound to other kinases. 1E8W (*D*, quercetin in complex with phosphatidylinositol 3-kinase γ) and 1H01 (*E*, fisetin in complex with CDK6) represent orientation I, whereas 2HCK (*F*, quercetin in complex with HCK) exemplifies orientation II. All pictures show residues that form hydrogen bond with the inhibitors (Lys[67], Glu[89], Glu[121], and Asp[186]). In the PIM1 structures, the three residues near the ATP-binding site that differentiate PIM1 from PIM2 (Ser[64], Glu[124], and Val[126]) are also shown. The inhibitors are colored by atom type: *red*, oxygen atoms; *yellow*, carbon atoms. *Dashed purple lines*, hydrogen bonds.

Specificity is always a concern with ATP pocket ligands. There are probably no absolutely selective inhibitors for a kinase but rather ligands that show a spectrum of affinities for their various targets. We have shown that quercetagetin is seven-fold more active against PIM1 than against eight other serine-threonine kinases and a tyrosine kinase, either with *in vitro* assays or in cell cultures. Interestingly, quercetagetin showed 10-fold more selectivity for PIM1 than for the homologous PIM2 kinase (sequence identity 96%). The ATP-binding pockets of these two kinases are identical with the exception of three residues along the edge of the PIM1 ATP-binding pocket—Ser[64] (Ala[65] in PIM2), Glu[124] (Leu[110] in PIM2), and Val[126] (Ala[112] in PIM2). Val[126] of PIM1 makes direct van der Waal's contact with the A ring of quercetagetin (Fig. 3A). Loss of such a contact due to the Val-to-Ala substitution is likely a contributing factor to the reduced activity of the compound in PIM2. The other residues are located close to the hinge Arg[122] (Arg[108] in PIM2). The polar side chains of Ser[64] and Glu[124] can form hydrogen bonds with Arg[122], thus affecting its conformation. Substitutions of these residues to hydrophobic amino acids in PIM2 will change the local environment (Fig. 3A).

The only large-scale examination of the specificity of flavonoid kinase inhibitors was reported recently by Fabian et al. (8). This investigation used a competitive binding assay to predict the inhibitor potency and specificity of the test agents. Flavopiridol was tested for binding affinity to 119 kinases. Twenty-three kinases bound flavopiridol under the test conditions, with binding constants ranging from 0.019 to 6.6 μmol/L. Interestingly, the tested cyclin-dependent

kinases bound flavopiridol less well than did calcium/calmodulin–dependent protein kinase kinase I. These data suggest that cyclin-dependent kinases may not be the only kinases inhibited in cells by flavopiridol. Both PIM1 and PIM2 were among the bound kinases, with binding constants of 0.52 and 0.65 μmol/L, respectively. Although there is no absolute correlation between binding constants and enzymatic activity, flavopiridol could conceivably inhibit the activity of both PIM1 and PIM2 in test systems.

Because quercetagetin has not been tested against a large number of other kinases, we cannot predict what other enzymes would be perturbed by this flavonoid. It is likely, however, that its spectrum of selectivity will be substantially different from that of flavopiridol. Quercetagetin showed clear preference for inhibiting PIM1 over PIM2, whereas flavopiridol did not. Furthermore quercetagetin inhibited the activity of the Aurora-A kinase (IC_{50}, ~4 μmol/L), a kinase that did not bind flavopiridol (8). The substantial



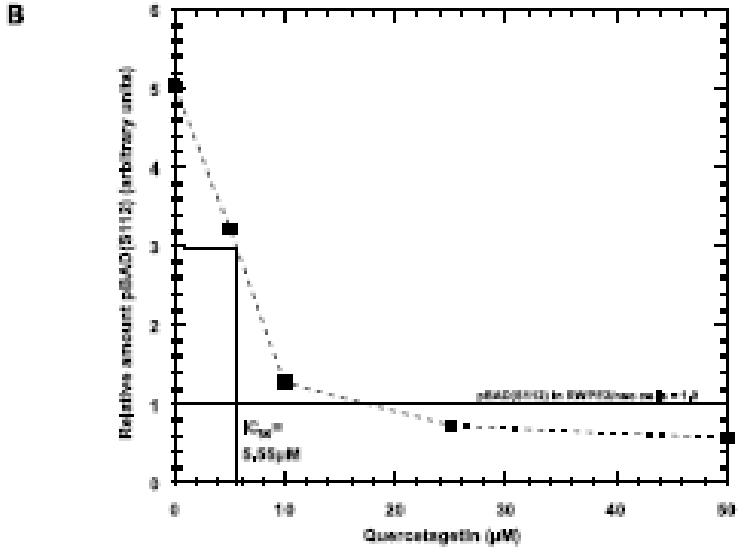


Figure 4. Quercetagetin inhibits PIM1 kinase activity in intact cells. A, RWPE2/neo or RWPE2/hpim1 cells were cultured in unsupplemented keratinocyte medium overnight, then treated with quercetagetin (0–50 μmol/L) for 3 h. Lysates were then prepared and examined by immunoblotting with the indicated antibodies. B, quantitation of the pBAD(S112)/actin ratio in immunoblots by using densitometry on the digital file. ID_{50}, 5.55 μmol/L.

homology between Aurora-A kinase and PIM1 kinase likely contributed to the low-level inhibitory activity of quercetagetin for the former; Aurora-A and PIM1 are 29% identical over their entire kinase domains; and the ATP binding pockets have 68% conserved amino acids.

An earlier, smaller-scale study looked at the effect of the flavonol quercetin on the *in vitro* kinase activity of 25 kinases, none of which were pim family kinases (29). At the tested concentration (20 μmol/L), quercetin inhibited the enzymatic activity of eight of the kinases. The propensity of this flavonol to form aggregates in aqueous solution has been advanced as an explanation for its widespread enzyme-inhibitory activity *in vitro* (30). We have not detected quercetagetin aggregates at concentrations of <10 μmol/L in aqueous solution, using a light-scattering assay (data not shown). Thus, we feel that this artifact does not account for the ability of this flavonol to inhibit PIM1 at nanomolar concentrations.

Because of the potential ambiguities that may accompany the use of small-molecule kinase inhibitors, a series of standards have been proposed for their use (29). To validate the results, it is desirable to show that the effects of an inhibitor disappear when a drug-resistant mutant of the protein kinase is overexpressed. Although convincing, this standard often fails due to the lack of an identified mutant with the desired properties. No such mutant has been identified for any of the pim kinases. Another potential standard is to show that the cellular effect of the drug occurs at the same concentrations that prevents the phosphorylation of an authentic physiologic substrate of the protein kinase. We have seen in these studies that half-maximal growth inhibition of prostate cancer cells occurred at a drug concentration (3.8 μmol/L) that approximated the IC_{50} for PIM1 enzyme inhibition in cells (5.5 μmol/L). Furthermore, the selectivity for prostate cancer growth inhibition, in proportion to endogenous PIM1 levels, was greatest at 6.25 μmol/L. Higher concentrations suppressed growth more, but the relationship to endogenous PIM1 levels was obscured. These data suggest that, at relatively low concentrations (perhaps 5–10 μmol/L), the growth-inhibitory effects of quercetagetin likely involve PIM1 antagonism. A third standard is to observe the same effect with at least two structurally unrelated inhibitors of the protein kinase. Previously described inhibitors of pim

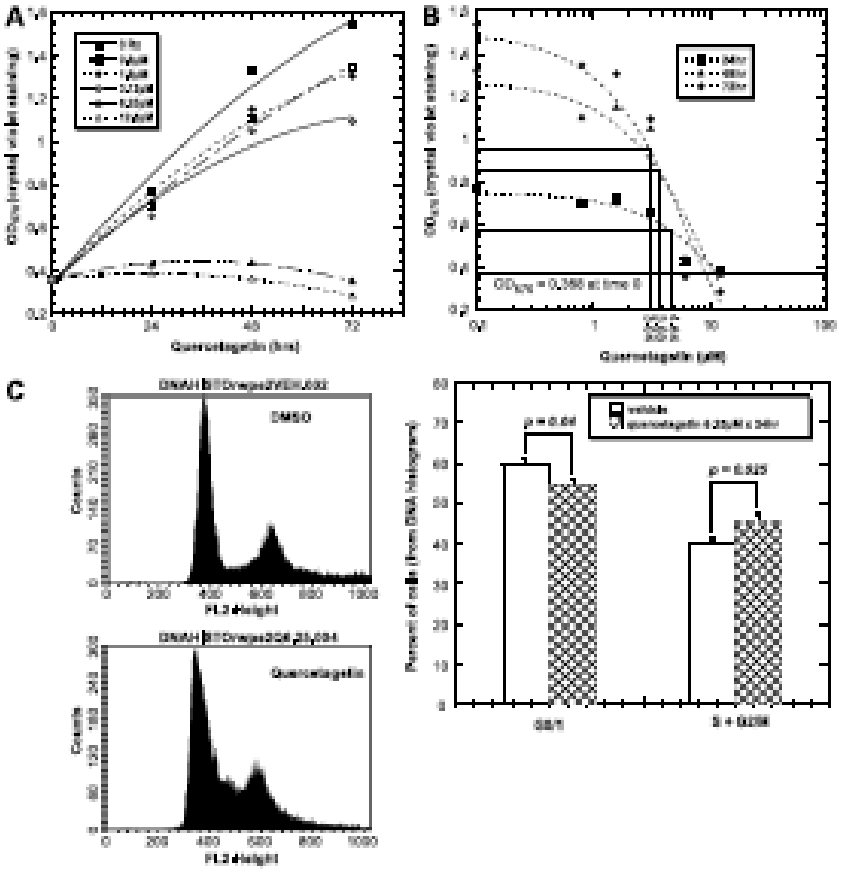


Figure 5. Quercetagetin inhibits growth of prostate cancer cells at concentrations that inhibit PIM1 kinase activity. *A*, growth curve of RWPE2 cells with different concentrations of quercetagetin. Cell number is measured by crystal violet staining. *Points*, mean of triplicate determinations from one of four similar experiments. *B*, calculation of ED_{50} at 24, 48, and 72 h of drug exposure. Average ED_{50} from all curves is 3.8 μmol/L. *C*, DNA histograms from RWPE2 cells treated with vehicle or quercetagetin 6.25 μmol/L × 24 h. Proportion of cells in G_0-1 or S + G_2-M fractions. *Columns*, mean of triplicate determinations from three independent experiments; *bars*, SD. *P* values show the probability of no difference by *t* test.

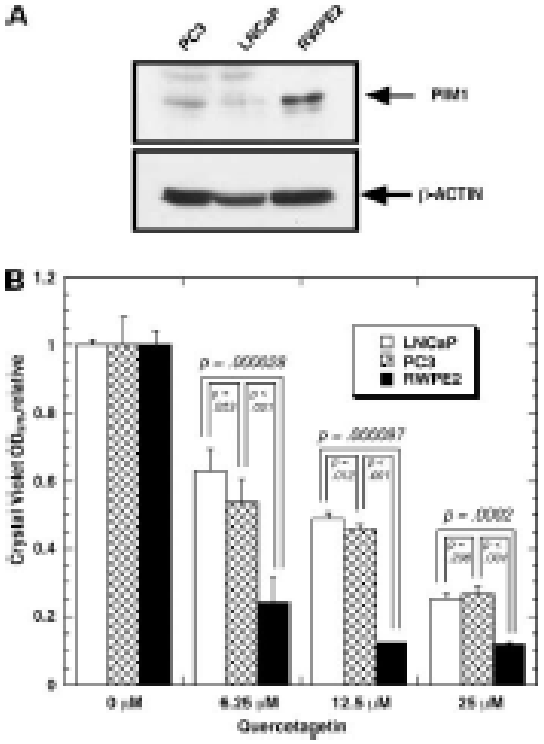


Figure 6. Quercetagetin inhibits the growth of prostate cancer cells in proportion to their content of PIM1. *A*, measurement of intracellular PIM1 in PC3, LNCaP, and RWPE2 prostate cancer cells by immunoblotting. *B*, growth inhibition by treatment of prostate cancer cells with quercetagetin for 72 h. *Columns*, mean of triplicate determinations from one of two similar experiments; *bars*, SD. *P* values were calculated by paired *t* tests and represent the probability that there is no difference between the two compared populations.

kinases are either less active or less specific flavonoids (7, 9), the same structural class as quercetagetin, or staurosporine analogues (8, 9, 21). We therefore used small interfering RNA as a genetic means to identify a *pim-1*–dependent phenotype. Proliferation of prostate cells was suppressed with both the genetic and chemical inhibitors of PIM1 activity. These data show that quercetagetin is an authentic small-molecule inhibitor of PIM1 kinase.

The crystal structures of PIM1 complexed with quercetagetin, myricetin, and 5,7,3′,4′,5′-pentahydroxyflavonone show that flavonoids bind to PIM1 in two distinct orientations. Although interesting, this is not a surprising observation, as flavones have shown a variety of binding modes in kinases (9, 22, 23, 26–28). An examination of the intermolecular interactions of each flavonoid with PIM1 does not clearly reveal why one orientation was adopted over the other. However, it is possible that the presence of three hydroxyl groups on the B ring of myricetin and 5,7,3′,4′,5′-pentahydroxyflavone discourages these two flavonoids from adopting the binding orientation observed for quercetagetin. The hydrophobic side chain of Leu^{120}, which extends into the ATP pocket in the same region occupied by the B ring of quercetagetin (Fig. 3A), may be incompatible with the 5′ hydroxyl group of myricetin and 5,7,3′,4′,5′-pentahydroxyflavone.

Both *pim-1* and *pim-2* can phosphorylate 4EBP-1, a regulator of protein translation (31, 32). Rapamycin was unable to block this effect. These data suggest that *pim* kinases may function in a parallel pathway to the phosphatidylinositol 3-kinase/AKT/mammalian target of rapamycin cascade to regulate and support protein synthesis under stress conditions. Because AKT-1 and PIM2 function cooperatively to induce lymphoma formation in transgenic mice (6), it may be necessary to target both pathways for effective antitumor effects. Several prototype AKT inhibitors have been described (33, 34). Our identification of quercetagetin as a PIM1 inhibitor provides a tool for tissue culture studies to investigate this hypothesis. Under the tested conditions, we found no evidence that quercetagetin inhibited the phosphorylation of AKT on Ser^{473}. Thus, it may be possible to combine inhibitors of these kinases to detect additive or synergistic effects resulting from the blockade of the two kinase pathways.

References

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National Professional Responsibilities

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1987 Diagnostic Radiology
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Member, site visit team for program project

Dr. George Hahn, PI; Stanford University
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Dr. Bayard Clarkson, PI, Memorial-Sloan
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Special Local Responsibilities

Member, Scientific Review Subcommittee
SVAMC, 1993, 1994, 1997
Member, Research & Development Committee
SVAMC, 1994, 1995
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Board Development Committee, Leukemia & Lymphoma Society (Southern California Chapter), 2003

Consultant

Cetus Corporation (1986)
EncorePharma (2001-present)
Myriad Genetics (2002-present)
Exelixis Pharmaceuticals (2005-present)

GRANTS & CONTRACTS (PRINCIPAL INVESTIGATOR) *Note: This listing does not include multicenter clinical trials in which Dr. Lilly was the local principal investigator.*

National Institutes of Health F32CA27980 *Hyperthermia of animal and human tumors*; 7/80-6/82

National Institutes of Health R01CA18138-*11 Prediction of thermal tolerance by in vivo NMR spectroscopy*; 7/82-6/83

National Institutes of Health R01CA36790 *Assessment of hyperthermia by in vivo ^{31}P-NMR spectroscopy*; 9/84-9/87

Cetus Corporation *Characterization of a human granulocyte CSF*; 7/85-6/86

National Institutes of Health R01CA45672 *Cytokine signaling in myeloid leukemia*; 9/87-10/98

VA Merit Review Award *Non-protein hematopoietic agents*; 10/90-4/97

March of Dimes Birth Defects Foundation *Characterization of a 28kd protein related to G-CSF*; 7/93-6/96

Lymphoma Research Foundation of America *Mechanism of action of the pim-1 oncogene*; 7/95-7/96

Roche Pharmaceuticals *Preclinical study of Roferon and bryostatin 1 in a melanoma model*; 1/98-12/99

Department of Defense, National Medical Technology Testbed #76-FY99: *Cell-permeable proteins for cell regulation.* 12/99 – 7/02

Leukemia Society of American Translational Award *Propionic Acid Analogues for CLL*. 9/1/01 – 8/31/05

Celgene Corporation, *Phase I-II trial of combined GM-CSF (sargramostim) and thalidomide for hormone-refractory prostate cancer* (5/02-5/04).

National Institutes of Health R03CA107820 *Molecular Targets of NSAIDs in Prostate* Cancer; (5/1/04 – 4/30/07)

Department of Defense, CDMRP Prostate Cancer Program PC040635 *Pim-1: A Molecular Target to Modulate Cellular Resistance to Therapy in Prostate Cancer* (10/04 – 10/07)

Pharmion Corporation, *Use of azacytidine to reverse silencing of GST-p1 in early prostate cancer.* (10/05 – 10/07)

GRANTS and CONTRACTS (Co-investigator)

National Institutes of Health R01CA097043 *Molecular pathology of 2-deoxy-5-azacytidine*; L. Sowers, PI; Michael Lilly, co-investigator (10% FTE). 7/1/03 – 6/30/08

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Kirshbaum M, **Lilly M**: Multiple growth factors induce expression of the Bcl-2 protein in 32D murine hematopoietic cells, but differ in their ability to inhibit apotosis. *Blood* 84:423a, 1994.

Lilly M, Pettit G: Identification of the cephalostatins as potent cytotoxic agents for myeloid leukemia cells. *Blood* 86:517a, 1995 (poster presentation)

Lilly M, Kraft A, Rotman E: Enforced expression of the human 33kd Pim-*1* kinase enhances autonomous proliferation and tumorgenicity in factor-dependent murine FDCP1 cells. *Blood* 86:588a, 1995 (oral presentation).

Lilly M, Cooper JJ: Enforced expression of the human 33kd Pim-1 kinase prevents apoptosis-associated mitochondrial dysfunction and upregulates *bcl-2* mRNA expression in murine myeloid cells. (oral presentation, ASH 12/97)

Wu X, Molinaro C, **Lilly M**, Casiano C: Caspase-mediated cleavage of the transcription co-activator p75 during apoptosis (abstract #993). *Proc AACR* 41:155 (2000).

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Lilly M, Frankel AE, Salo J, Kraft AS: Distinct domains of the human GM-CSF receptor alpha subunit mediate activation of Jak/Stat signaling and differentiation (abstract #2455). *Blood* 96:572a (oral presentation, ASH 12/00)

Chen CS, **Lilly MB**, Wang FS, Howard FD, Houwen B: Rapid monitoring of peripheral blood stem cells (PBSC) mobilization by using cell membrane phospholipid content correlates well with CD34+ measurements, successful harvest and engraftment (abstract #1642). *Blood* 96:380a (poster presentation, ASH 12/00)

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Kastaros EP, Casiano C, Colburn KK, **Lilly M**, Weisbart RH, Kim J, Green LM: Lupus associated anti-guanosine antibodies: potential pathogenic effects. *Arthritis & Rheumatism* 44:S99 (2001)

Yan B, Zemskova M, Kraft AS, Koskinen PJ, **Lilly MB**: The pim-2 kinase phosphorylates Bad on serine-112 and reverses Bad induced cell death. (abstract #2919). *Blood* (poster presentation, ASH 12/02).

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